Identification of Autoantigens in Psoriatic Plaques Using Expression Cloning

David A. Jones, Nikhil Yawalkar, Ki-Young Suh, Sara Sadat, Benjamin Rich, and Thomas S. Kupper
Harvard Skin Disease Research Center, Department of Dermatology, Brigham and Women's Hospital, Boston, Massachusetts, USA

To search for autoantigens in psoriatic plaques, we screened cDNA libraries of plaque epidermis with psoriatic serum samples. This approach has been highly successful in identifying tumor antigens, but has not been widely applied to autoimmune disease. We identified 11 autoantigens including three with prominent reactivity and plausible disease relevance. These are keratin 13 (K13), heterogeneous nuclear ribonucleoprotein-A1 (hnRNP-A1), and a previously uncharacterized protein, FLJ00294. Serum antibody screening for these demonstrated reactivity in 40%, 38%, and 27% of psoriasis patients, respectively. Most positive samples reacted with all three, and we found that this was due to cross-reactivity among them. Enzyme-linked immunospot assay (ELISPOT) analysis of psoriatic peripheral blood T cells confirmed that these autoantigens are also recognized by T cells. This demonstrates that this is a feasible method to identify autoantigens in an autoimmune target tissue, and suggests that these antigens warrant further study in psoriasis. Furthermore, but peripheral blood of normal controls reacted to these autoantigens with essentially the same frequencies as patients, suggesting that psoriatics may have not only an immune system which is capable of reacting to certain autoantigens, but also to a skin immunoregulatory alteration which allows this normal reactivity to develop into abnormal inflammation.

Key words: autoantibodies/autoimmunity/human/skin
J Invest Dermatol 123:93–100, 2004

Current evidence suggests that a limited number of self-antigens are normally recognized by the immune system (Lacroix-Desmazes *et al*, 1998; Plotz, 2003), and that loss of proper regulation of reactivity to these antigens contributes to autoimmune disease (Bach *et al*, 1998; von Herrath and Harrison, 2003). Although numerous autoantigens have been described for certain organ-specific T-cell-mediated autoimmune diseases such as multiple sclerosis and diabetes (Mocci *et al*, 2000), many other autoimmune diseases have few or no associated autoantigens described. Furthermore, the mechanisms by which the autoreactive T cells lead to disease are very difficult to study because in the most widely studied diseases, tissue is not easily accessible. It would therefore be of considerable value to have a method capable of easily identifying autoantigens recognized by T cells, and to define a set of autoantigens in a common disease with easily accessible tissue. In this report, we present progress toward these goals. In order to identify autoantigens expressed in a target organ, we use an approach that has been very successful in the identification of antigens recognized by T cells in tumor immunity. The studies are done in psoriasis, which is an ideal model disease because it is common and tissue is readily accessible.

Identification of antigens recognized by T cells in human disease states has proved very difficult in the past, requiring laborious methods such as isolating reactive T-cell clones and eluting peptides from major histocompatibility complex molecules. A new approach in the field of tumor immunity has led to the identification of an enormous variety of T-cell tumor antigens over the past several years. This approach is termed SEREX[3], for SErological identification of Recombinant EXpressed antigens, and is based on the presence of both T- and B-cell reactivities to an antigen mediated through a common set of helper T cells (Tureci *et al*, 1997, 1999; Old and Chen, 1998). For these antigens, the parallel B-cell response may have no significant role in disease, but the existence of these antibodies can serve as a marker for a corresponding set of T cells that react to the same antigen. Since tumor immunity can be regarded as one form of T-cell-mediated autoimmunity, and since there is good experimental evidence for parallel B- and T-cell responses in autoimmune diseases, we reasoned (as have others; Krebs *et al*, 2003) that this approach could be used to identify autoantigens in a T-cell-mediated autoimmune disease. Our results demonstrate that this is a productive approach. Screening proteins in expression libraries of psoriatic plaque epidermis identified a limited subset (11 proteins) with high levels of autoreactive antibodies, supporting the idea that the number of proteins capable of serving as autoantigens is quite limited. Three of these have especially high-level reactivity and plausible relevance in psoriasis and psoriatic arthritis. We also verify using ELISPOT analysis that T cells respond to these proteins at levels that are similar to responses seen to established autoantigens in other diseases.

Abbreviations: hnRNP-A1, heterogeneous nuclear ribonucleoprotein-A1; K13, keratin 13; SEREX, SErological identification of Recombinant EXpressed antigens

Results

Library screening Libraries were screened using a combined mixture of five psoriatic serum samples. Positive plaques were purified by repeat rounds of plating, screening, and recovery of positive phage. Inserts were amplified by PCR and sequenced, and each positive phage was then tested for reactivity with up to 19 separate psoriatic serum samples in a mixed plaque assay (see Table 1). Three libraries were screened: two created from psoriatic plaque epidermis and a commercially available cultured neonatal foreskin keratinocyte library. Approximately 2×10^6 plaques were screened from each library using the psoriatic serum samples. This represents screening of approximately one fifth of the total complexity of the libraries since complete immunoscreening requires testing approximately ten times the number of independent clones in the library. Based on the library screening results, three antigens were selected for further study using a larger number of psoriatic serum samples and normal control samples.

Antibody responses against antigens Table 2 presents the results of mixed plaque assays for 48 psoriatic subjects and 28 normal controls. This assay gives a semiquantitative measure of the antibodies to each protein in each serum sample, which we have graded on a scale of $+$ to $++++$ (Fig 1 shows examples of the appearance of this type of blot, with 1*A* graded as $++++$ and 1*B–D* graded as $+$). As shown in columns 1–3 of Table 2, 40% of psoriatics have autoantibodies that react to K13, 38% to hnRNP-A1, and 27% to FLJ00294. A prominent feature of this data set is that subjects reacting strongly to one antigen generally react to all three antigens. This interesting finding can be explained by either of two possibilities: (1) some people happen to have autoreactive antibodies to all three of these proteins, or (2) the antibodies to these proteins cross-react. An experimental artifact appears unlikely because western blot results using purified recombinant K13 and hnRNP-A1 confirm the specificity of all strongly reactive serum samples, with rare reactivity in samples negative in the mixed plaque assay (columns 4 and 5 of Table 2). Very similar data was found for normal controls. Of the 28 normal controls tested, 39% react to K13, 32% to hnRNP-A1, and 29% to FLJ00294. The finding that strongly reactive serum samples react to all three antigens was also seen for normal controls. Western blot results again confirmed the band specificity of strongly reactive serum samples.

The mixed plaque assay was chosen to test for autoantibodies because antigens generally fold well as β-galactosidase fusion proteins and are minimally denatured in the blotting process, and because no additional subcloning is required. It also has a proved track record in SEREX studies (Sahin *et al*, 1995; Robinson *et al*, 2000). This was found to be considerably more sensitive than the western blot assay for these proteins, with easily discernable dark spots on the phage blots but weak bands on the western blots. This suggests that these epitopes are conformation dependent and that sensitivity is lost in the denaturation of SDS-PAGE. Consistent with this, all highly reactive serum samples in the mixed plaque assay were also positive in the western blot, whereas many of the weakly positive serum samples in the mixed plaque assay were not positive in the western blot. Also, several subjects are positive in the western blot but not in the mixed plaque assay (e.g., P27 in Table 2), which likely represents the unmasking of irrelevant epitopes by SDS-PAGE and suggests that the western blot also has lower specificity compared with the mixed plaque assay. We also tried testing humoral responses in an ELISA format using the purified recombinant proteins (data not shown). This was complicated by problems with trace contaminating *E. coli* proteins not visible with Coomassie staining but which sometimes produced prominent bands on western blot analysis.

T-cell responses against antigens Figure 2 shows the results of ELISPOT analysis. PBMC were available from 25

Table I. Insert sequencing and initial mixed plaque assay results for reactive phage

Phage insert sequence	Accession number	Psoriatic serum
Keratin 13 (transcript variant 1)	NM_153490	6/19
Heterogeneous nuclear ribonucleoprotein-A1	NM_002136	6/19
Putative protein FLJ00294	AK122583	6/19
Bullous pemphigoid antigen 1 (BPAG1)	NM_001723	3/19
Adenocarcinoma antigen ART1	AF197954	2/19
Chromosome 9 sequence	AL354928	1/15
Putative protein FLJ10335	NM_018062	1/15
LIM protein (similar to rat protein kinase C-binding enigma)	BC008741	1/14
Hepatocellular carcinoma-associated antigen 59	AF218421	1/14
Quinone oxidoreductase (NQO2)	J02888	1/14
Chromosome 14 sequence	NM_138288	1/14

Three cDNA expression libraries in lambda phage were created from psoriatic plaque epidermis and screened with a mixture of five psoriatic serum samples. Reactive phage were plaque purified and inserts were sequenced (columns 1 and 2). The phage were then tested for reactivity with 14–19 individual psoriatic serum samples (column 3, reactive serum samples per total).

Table II. Analysis of antibody responses against autoantigens for psoriatic and normal subjects

Sample	K13 MPA	hnRNP-A1 MPA	FLJ00294 MPA	K13 WB	hnRNP-A1 WB
P1	+ + +	+ +	+ + +	+	+
P6				+	
P9	+	+	+		
P10	+ +	+ +	+	+	+
P11	+	+		+	
P12	+	+		+ +	
P15	+	+	+		
P16	+ +	+ +	+ +		+
P17	+	+			
P18	+ + +	+ + +	+ + +	+ +	+
P21	+ + +	+ + + +	+ + +	+	+
P22	+	+			
P24	+	+	+		
P26				+	
P27				+ + +	
P30	+	+	+		
P31	+	+			
P39	+ + + +	+ + + +	+ + +	+ +	+
P40	+	+		+ +	
P43	+ +	+	+	+	
P44	+	+	+		
P46	+ +		+		
26 other psoriatics	−	−	−	−	−
N1	+	+	+	+	
N2				+	
N4				+	
N6	+ +	+ +	+		
N9	+				
N12	+				
N15	+ +	+ +	+ +	+ +	
N16	+ + +	+ + +	+ + +	+	+
N21				+	
N22	+	+	+		
N23	+ + +	+ + + +	+ + +	+	+
N24	+ +	+ +	+	+	
N26	+	+			
15 other normals	−	−	−	−	−

Serum samples from 48 psoriatic subjects and 28 normal controls were tested for autoantibodies to K13, hnRNP-A1, and FLJ00294 in the mixed plaque assay (MPA). The same samples were also used in a western blot (WB) assay using purified recombinant K13 and hnRNP-A1. A blank cell indicates a negative result. Subjects with all negative results are grouped together. K13, keratin 13; hnRNP-A1, heterogeneous nuclear ribonucleoprotein-A1.

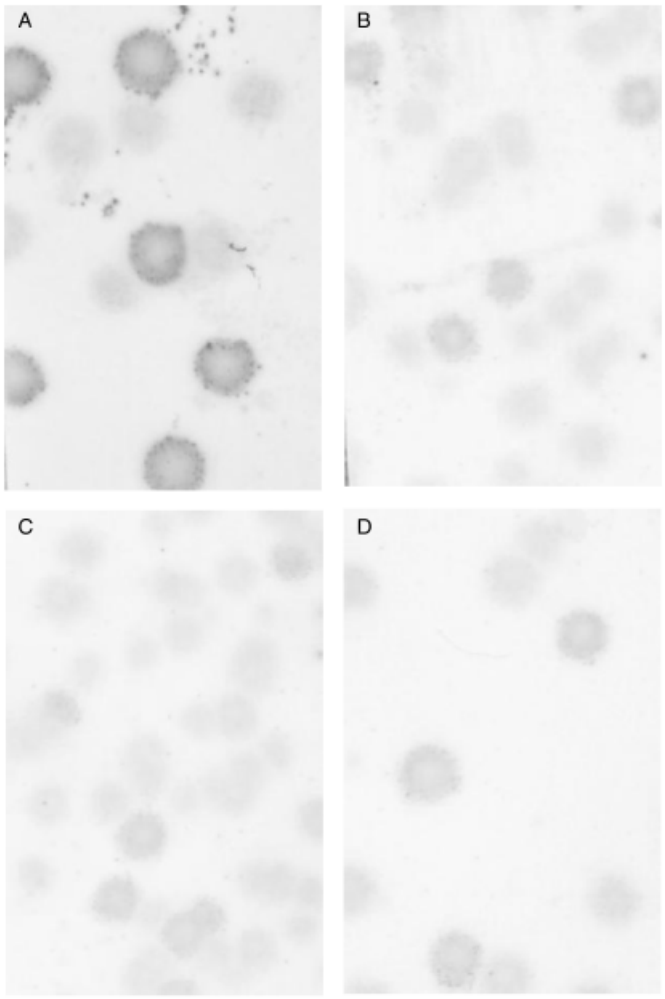

Figure 1
Antibody cross-reactivity among antigens. (*A*) A mixed plaque assay using pre-cleared serum sample P39 tested with an approximately 1:1 mixture of keratin 13 (K13) recombinant plaques and non-recombinant control plaques. This serum sample was then adsorbed to a sheet of confluent K13 plaques and bound antibodies were eluted and used to probe mixtures of (*B*) K13, (*C*) heterogeneous nuclear ribonucleoprotein-A1, (*D*) FLJ00294 plaques with non-recombinant plaques. Each assay is internally controlled with the intermixed non-recombinant plaques, which appear fainter and less distinct than the antigen-expressing plaques.

of the normal subjects and 16 of the psoriatic subjects. These data have been separated from the data in Table 2 and are presented in graphical format for clarity and comparison with other similar results. The first four columns show the numbers of T cells reactive to K13 and hnRNP-A1 in each PBMC sample. The positive control recall antigen, tetanus toxoid, gave very strong responses in most subjects (columns 5 and 6), whereas most subjects had very few spontaneously reactive cells (columns 7 and 8). The FLJ00294 protein proved difficult to obtain in sufficient quantity and purity for the ELISPOT assays and has not yet been tested. Comparison of T-cell reactivity of each subject with antibody reactivity did not yield any significant correlation (data not shown).

Cross-reactivity among antigens Figure 1 shows results of experiments that demonstrate cross-reactivity of the

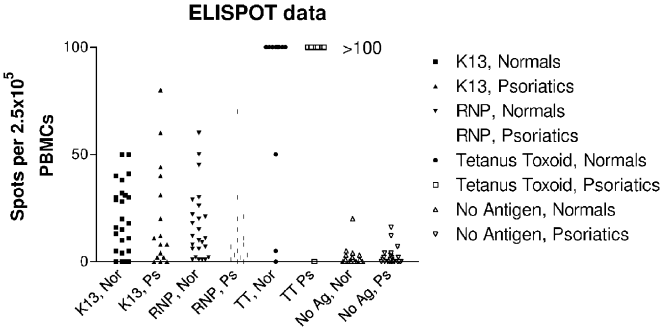

Figure 2
ELISPOT testing of keratin 13 (K13) and heterogeneous nuclear ribonucleoprotein-A1 (hnRNP-A1). Peripheral blood mononuclear cells (PBMC) from psoriatic or normal subjects were incubated with PAGE-purified recombinant antigen and spots formed by activated IFN-γ secreting cells were counted. Each symbol on the graph represents one subject.

antibodies among all three antigens. Figure 1*A* shows the strong serum reactivity of subject P39 to K13 in a mixture of K13 and negative control plaques. Antibodies from this subject's serum were bound to large plaque lifts of K13-expressing phage, eluted, and used to probe K13, hnRNP-A1, and FLJ00294 mixed plaque blots (Figs 1*B–D*). Assays using antibodies eluted from hnRNP-A1- and FLJ00294-expressing phage (not shown) produced weak but clear reactivity on mixed plaque lifts of all three antigens that was very similar to those shown. Assays using antibodies eluted from non-recombinant phage showed no specific reactivity, as expected (not shown). We also tried reacting the eluted antibodies to western blot strips containing the three recombinant proteins, but found that specific bands could not be reliably discerned, which we believe is due to the loss of sensitivity of the western blot relative to the mixed plaque assay as was discussed above.

Discussion

Current evidence suggests that a limited number of self-antigens are normally recognized by the immune system (Lacroix-Desmazes *et al*, 1998; Plotz, 2003). This repertoire of autoreactivity varies somewhat among individuals, but overall is highly conserved, even across species, and responses to these antigens remain stable throughout an individual's life. The total number of these autoantigens is estimated to be in the low hundreds of proteins (Plotz, 2003). Responses to some autoantigens are reasonably specific for disease, but in other cases reactivity is not specific and in these cases, loss of proper regulation of normal autoimmune reactivities along with elements of an altered inflammatory response in target organs are postulated to lead to autoimmune disease. The details of this, however, remain elusive, especially for the autoimmune diseases mediated predominantly by T cells (Bach *et al*, 1998; von Herrath and Harrison, 2003).

Our results demonstrate that the SEREX approach is useful in the study of organ-specific T-cell-mediated autoimmune disease by defining candidate autoantigens. Primary screening revealed a limited number of antigens

(11) after screening approximately one-fifth of the total library complexity, and a large fraction of subjects tested (up to 40%) react to these. This supports the concept that there is a very limited subset of proteins in a tissue that are capable of serving as autoantigens, and that this subset is similar among individuals (Lernmark, 2001; Plotz, 2003). We estimate that in future work, full screening of the libraries should be capable of identifying a relatively complete repertoire of autoantibodies to components of psoriatic plaque epidermis. One initial concern was interference by autoantibodies to common proteins, such as anti-actin autoantibodies or antibodies to heat shock proteins. Another concern was the possibility that autoantibody responses could be regulated to levels that are low compared with tumor antigens or exogenous antigens. We chose a 1:500 serum dilution for library screening as is commonly done for tumor antigen SEREX, and found that a subset of antigens with easily discernable reactivity could in fact be identified.

Three antigens identified in the primary screening appear promising based on high-level reactivity and plausible disease relevance. K13 is an interesting antigen in several respects. First, it has significant homology with K17, which was previously identified as candidate autoantigen based on the presence of both antibodies and T cells that cross-react with a streptococcus M protein (Gudmundsdottir *et al*, 1999). Second, K13 is not present in adult skin normally, but is present in fetal skin (van Muijen *et al*, 1987), and is upregulated in regenerating epidermis (Kallioinen *et al*, 1995). This could potentially provide an explanation for the Koebner phenomenon, in which lesions of psoriasis develop in skin that has been traumatized. Third, K13 is present in large amounts in non-keratinizing squamous epithelium such as the tongue, esophagus, and cervix, and could therefore be related to geographic tongue, which is thought to be a localized psoriasis variant. Similarly, K13 is present in genital and perianal epidermis (van Muijen *et al*, 1986; Stoler *et al*, 1988) that are affected areas in the so-called inverse psoriasis. Finally, expression of K13 is regulated by retinoids (van Rossum *et al*, 2000a, b), and although this has not been studied in the context of psoriasis, it could potentially be related to the efficacy of some retinoids in the treatment of psoriasis.

Heterogeneous nuclear ribonucleoprotein A1 (hnRNP-A1) also has several interesting features as a candidate autoantigen in psoriasis. It is a component of the spliceosome composed of three domains: two RNA-binding domains and a carboxy terminal domain that is glycine-rich and homologous to basic keratins. Although most anti-RNP antibodies in connective tissue diseases bind to the RNP-binding domains, there is a subset of rheumatoid arthritis patients with antibodies to the keratin-homology domain of hnRNP-A1 (Astaldi-Ricotti *et al*, 1989). This subset of patients was further found to have anti-keratin antibodies that cross-react with hnRNP-A1 and could be removed by adsorption to purified hnRNP-A1 (Montecucco *et al*, 1990). Our experiments were not designed to detect a correlation between hnRNP-A1 reactivity and psoriatic arthritis, but the possibility of such a relationship is intriguing. Another previously demonstrated cross-reactivity of hnRNP-A1 is HTLV-1 tax protein (Levin *et al*, 2002). No association between psoriasis and HTLV-1 infection is known, but infection with a related retrovirus producing a protein similar to tax is an intriguing possibility in the pathogenesis of psoraisis. These experiments were in fact initially developed to account for the possibility of viral antigens as well as autoantigens. The method only requires that the antigen be expressed as mRNA in the target tissue, not that it necessarily be a self-antigen.

The FLJ00294 insert nucleotide sequence matches exactly to chromosome 8p11 sequence and the GENBANK entry for the putative protein FLJ00294. NCBI BLAST searching revealed that this protein is homologous to rat and rabbit Rab interacting proteins, and also revealed a match with K17. The significance of the homology with K17 remains to be verified since the corresponding peptides have two amino acid mismatches: EGEDALVTQY for FLJ00294 and EGEDAHLTQY for K17. Although both RNP and FLJ00294 show some sequence homology to keratins in general, it is important to note that analysis of the antigenic insert of each clone does not indicate a single region of homology common to K13, hnRNP-A1, and FLJ00294. As a result, it can be concluded that the antibody cross-reactivity is likely to involve secondary structure (which is supported by the higher sensitivity of the mixed plaque assay relative to western blotting), and that some amount of epitope spreading may be involved.

Screening of K13, hnRNP-A1, and FLJ00294 with larger numbers of serum samples (Table 2) demonstrates that high percentages (up to 40%) of subjects react to these, and that subjects with high-level antibodies appear to have cross-reactive antibodies. This cross-reactivity was then confirmed directly by demonstrating that antibodies eluted from K13 blots react with hnRNP-A1 and FLJ00294 (Fig 1). These findings are strikingly similar to previous studies of autoantigens identified in autoimmune thyroid disease. Testing of large numbers of subjects for antibodies to thyroglobulin and thyroid peroxidase showed that most subjects with high-level autoantibodies to one antigen have autoantibodies to the other (Pedersen *et al*, 2003), and direct cross-reactivity has been shown for thyroglobulin and thyroid peroxidase using antibody elution experiments similar to those performed here (Ruf *et al*, 1992). Also of potential relevance is the finding that autoreactivity to these thyroid antigens is an inherited trait. If such inheritance also proves to be the case with these candidate autoantigens in psoriasis, it could be related at least in part to the genetic predisposition in psoriasis.

As is seen with many other autoantigens that have been studied in T-cell-mediated autoimmune diseases, these candidate autoantigens have similar antibody reactivity in both patients and controls. It will therefore require more detailed experiments to demonstrate the exact role of these candidate autoantigens in psoriasis. One approach that appears promising is to identify a more complete set of autoantibodies with additional screening. In the case of diabetes, where numerous autoantigens have been identified previously, it has been found that autoantibodies are not disease specific when tested individually, but the simultaneous presence of multiple antibodies has high specificity for disease (reviewed in Leslie *et al*, 1999, see also Maclaren *et al*, 1999). A more complete set of

autoantigens in psoriasis might also demonstrate relationships to disease prediction and classification as seen in other diseases (Leslie *et al*, 2001).

One technical consideration in this application of SEREX is the method used after initial screening to test larger groups of serum samples. We found that the mixed plaque assay, although requiring tedious serum pre-clearing, is highly robust and very sensitive because it minimizes antigen denaturation. Alternatives include an ELISA or western blot using purified protein, and we tried each of these. The ELISA is commonly used in tumor antigen SEREX because it is quantitative and convenient. But we found the ELISA unsatisfactory for measurement of these autoantibodies due to trace *E. coli* contaminants in the purified proteins. Western blotting avoids this problem by verifying reactivity to a specific band, but we find that the denaturation of SDS-PAGE decreases sensitivity for these autoantibodies to an unacceptable degree. The conformation dependence and relatively low antibody concentrations we find here suggest that, as has been found for most antibody studies in diabetes and multiple sclerosis, radioimmunoassay may be the best approach for screening large numbers of samples in the future (Kawasaki and Eisenbarth, 2000). These issues may also place constraints on possible alternative systems for probing autoantibody repertoires (Hueber *et al*, 2002; Robinson *et al*, 2002).

Finally, our ELISPOT results confirm that these autoantigens can also be recognized by peripheral blood T cells. This is an important validation of the approach of using antibodies as surrogate markers to screen for autoantigens in T-cell-mediated autoimmune disease. Both the frequency of positive responses and the magnitude of responses are comparable with previous work with autoantigens in multiple sclerosis (McCutcheon *et al*, 1997; Pelfrey *et al*, 2000; Hellings *et al*, 2001) and diabetes (Alleva *et al*, 2001). We had hoped for a correlation between antibody levels and T-cell responses for each subject, but none was found. The lack of such a correlation, however, has also been seen in diabetes (Hummel *et al*, 1996; Narendran *et al*, 2003), as has an inverse correlation in one instance (Harrison *et al*, 1993).

Studies of autoreactive T cells in other autoimmune diseases have generally shown similar reactivity of peripheral blood T cells between patients and normal controls. This is taken as evidence that it is insufficient to simply recognize a self-antigen, and that a dysregulated inflammatory response within the target organ is critical for the development of autoimmune disease (von Herrath and Harrison, 2003). Our studies also demonstrate similar peripheral blood reactivity among psoriatics and normal controls, and therefore suggest that this concept applies to psoriasis as well. Studies of cerebral spinal fluid (CSF) T cells in multiple sclerosis (Chou *et al*, 1992; Zhang *et al*, 1994) and lesional T cells in animal models of autoimmune disease do show disease specificity in response to autoantigens. We therefore anticipate that in future work, the accessibility of lesional tissue in psoriasis will allow studies of target organ damage that are currently not possible in other human diseases. Other studies that may prove useful in future work are peptide mapping to identify immunodominant peptides of higher specificity than whole proteins (Kerlero de Rosbo *et al*, 1997; Pelfrey *et al*, 2000; Alleva *et al*, 2001; Tejada-Simon *et al*, 2001), and the use of anti-CD28 blocking antibody to show a difference in the activation state of antigen-specific T cells in patients relative to controls (Lovett-Racke *et al*, 1998; Viglietta *et al*, 2002).

In summary, we have utilized an expression cloning approach to identify novel autoantigens from libraries of psoriatic plaque epidermis. This approach is based on the SEREX method widely used to identify tumor antigens, and appears to be a very practical alternative to other methods of identifying antigens for T cells in autoimmune disease. Three autoantigens that we have identified have plausible relevance in psoriasis as well as patterns of antibody and T-cell reactivity very similar to those seen in other autoimmune diseases. Further study of these autoantigens should provide significant additional insight into the pathophysiology of psoriasis, and study of psoriasis may in turn yield insights into other autoimmune diseases through experiments that are practical only in this common skin disease.

Materials and Methods

Patients All blood and biopsy samples were obtained following informed consent according to an IRB-approved study protocol. Psoriasis patients were eligible if they had active plaque-type psoriasis regardless of severity. No patients received systemic or ultraviolet light treatment for more than 1 mo prior to obtaining a blood sample for antibody testing. Shave biopsies for library production were obtained from untreated plaques. No patients had an active or previous concominant diagnosis of bullous pemphioid.

cDNA library construction A cDNA library of cultured neonatal foreskin keratinocytes in the lambda gt11 vector was purchased from BD Biosciences Clontech, Palo Alto, California. Other libraries were constructed in our laboratory as follows. Shave biopsies of psoriatic plaque epidermis (approximately 1.5 cm^2) were obtained and total RNA was isolated using Trizol (Invitrogen, Carlsbad, California). Full-length cDNA was created and amplified using 10–13 cycles of PCR as described previously (Lukyanov *et al*, 1997). To improve stability and expression of the clones, cDNA was digested with *Rsa*I to obtain shorter cDNA fragments (average of 500 bp). This was followed by ligation of *Eco*RI adapters, ligation into lambda ZAP II arms (Stratagene, La Jolla, CA), packaging, and library expansion using standard procedures. Libraries had between 0.8 and 1.2 $\times$ 10^6 independent clones with 70%–80% recombinants. The presence of β-actin-expressing clones was verified by using a monoclonal antibody (Zymed, South San Francisco, California) for immunoscreening.

cDNA library screening Libraries were screened using standard immunoscreening protocols. In brief, isopropyl-beta-D-thiogalactopyranoside phosphate/nitro-blue tetrazolium-induced (Sigma, Sigma-Aldrich, St. Louis, Missouri) plaque lifts on nitrocellulose membrane (Stratagene) were blocked using 5% non-fat dried milk (NFDM) in Tris-buffered saline with 0.05% Tween 20 (TBST), washed with TBST, then incubated with serum samples diluted in TBST overnight at 4°C. Serum from five psoriasis patients was precleared extensively using plaque lifts of non-recombinant phage and combined at 1:500 dilution each. The secondary antibody was goat anti-human IgG conjugated to alkaline phosphatase (Southern Biotechnology #2040, 1:2000 dilution in TBST) and membranes were developed using 5-bromo-4chloro-3indoxyl solution (Spectrum, New Brunswick, New Jersey). Positive spots were marked and the corresponding phage plaques were recovered for repeat rounds of plaque purification. Inserts of plaque purified phage were sequenced by using PCR to create insert amplicons with T3 and T7 primers for inserts in lambda ZAP II or lacZ-specific flanking

primers for lambda gt11 (New England Biolabs, Beverly, Massachusetts).

Mixed plaque assay Recombinant and control phage were mixed 1:1, plated at low density, blotted and screened as for the library screening. After development, if dark and light plaques could not be clearly differentiated a negative result was assigned. Positive results were scored visually by two blinded investigators based on the difference in intensity between dark and light plaques using a scale of + (weak), ++, +++, or ++++ (very strong). As examples, Fig 1*A* would be graded as ++++, and Figs 1*B–D* would be graded as +.

Recombinant proteins Antigenic inserts from the libraries were partial length fragments ranging from 200 to 1200 bp. The antigenic hnRNP A1 fragment (bases 447–950 of the 962 base cDNA) covered almost the entire C-terminal keratin-homology domain and a small portion (84 bp) of the RBD2 domain, and was large enough for expression in *Escherichia coli*. The keratin 13 (K13) fragment (bases 1113–1376 of the 1376 bp cDNA) was too small for expression so the full-length sequence was amplified from psoriatic plaque RNA by RT-PCR and verified by direct sequencing. The FLJ00294 phage insert (bases 607–1385 of the partial length mRNA sequence available in accession AK122583) was large enough for expression in *E. coli*. All inserts were subcloned into the pQE-32 vector (Qiagen, Valencia, California) to attach an N-terminal 6 × histidine tag, and purified over Ni-NTA (Qiagen) using the manufacturer's protocols. Purified proteins were checked for purity using SDS-PAGE and Coomassie staining. In all cases, the major band was present at the correct molecular weight, but additional lower molecular weight bands were also present. These proved either degradation or pre-mature truncation products when tested by western blot staining with anti-RGS-his tag antibody (Qiagen).

Western blot Recombinant proteins were separated using SDS-PAGE, blotted onto polyvinylidene difluride membranes, blocked with 5% NFDM in TBST, washed, and cut into strips. Strips were incubated with serum samples diluted 1:50 in TBST with 1% NFDM for 2 h at room temperature. Strips were developed as above using secondary goat anti-human IgG-AP antibody incubation for 1 h and BCIP/NBT solution. Developed membranes were then assessed visually by two blinded investigators for the intensity of bands at the correct molecular weight. Semiquantitative grading of results for the western blot was performed as for the mixed plaque assay (+ to ++++).

ELISPOT PVDF ELISPOT plates (Millipore, Billerica, Massachusetts) were coated with anti-IFN-γ antibody (BD BioSciences Pharmigen, Palo Alto, California, clone NIB42) and blocked with 2% bovine serum albumin in TBST. Peripheral blood mononuclear cells (PBMC) were purified by centrifugation through Ficoll–Hypaque, washed, and resuspended in RPMI-1640 with 10% human serum AB and penicillin/streptomycin/glutamine supplementation. Purified recombinant antigens were added to 10^6 PBMC in 400 μL medium at 1 μg per mL for a 24 h pre-incubation period in round-bottom polypropylene tubes for antigen presentation. Tetanus toxoid was purchased from the Massachusetts Biologic Laboratories (Jamaica Plain, Massachusetts) and used at 1 μg per mL. PBMC were then resuspended and transferred to the plates at 2.5×10^5 PBMC/well in quadruplicate. After a 24 h period for IFN-γ secretion, plates were developed using a biotinylated second anti-IFN-γ antibody (BD Pharmingen, clone 4S.B3), streptavidin-alkaline phosphatase (BD Pharmingen), and BCIP/NBT solution according to standard protocols. Spots were counted manually by two blinded investigators, averaged, and then averaged over the four replicate wells to give the number of spots per 2.5×10^5 PBMC.

Antigen cross-reactivity experiments Three aliquots of serum from a psoriatic subject with high-level antibody reactivity (P39) were diluted 1:25 with TBST and pre-cleared extensively using plaque lifts of non-recombinant phage. The serum aliquots were then incubated with plaque lifts of high-density K13-, hnRNP-A1-expressing plaques, or non-recombinant plaques. These plaque lifts were then washed with TBST and incubated for 5 min in stripping buffer (0.1 M glycine, 0.15 M NaCl, pH 2.75), which was collected and immediately neutralized. The resulting antibody-containing solutions were then incubated with mixed plaque lifts as in the other mixed plaque assays.

Dr Catherine Wu of Dana-Farber Cancer Institute (Boston, Massachusetts) provided assistance and helpful discussions, and Drs Ivor Caro and Charles Taylor along with the staff of the Phototherapy Unit of Massachusetts General Hospital (Boston, Massachusetts) provided assistance in recruitment of study subjects. This work was supported by grants from the National Psoriasis Foundation and the American Skin Association (D. A. J.), the Swiss Foundation of Medical-Biological Scholarships and the Novartis Foundation (N. Y.), and the National Institutes of Health grants 5P30AR42689 and 5U19AI046130 (T. K.).

DOI: 10.1111/j.0022-202X.2004.22709.x

Manuscript received December 4, 2003; revised January 23, 2004; accepted for publication February 14, 2004

Address correspondence to: David A. Jones, Department of Dermatology, Harvard Skin Disease Research Center, Brigham and Women's Hospital, Harvard Institutes of Medicine Building, Room 660, 77 Ave. Louis Pasteur, Boston, Massachusetts 02115, USA. Email: dajones@partners.org

References

Alleva DG, Crowe PD, Jin L, *et al*: A disease-associated cellular immune response in type 1 diabetics to an immunodominant epitope of insulin. J Clin Invest 107:173–180, 2001

Astaldi-Ricotti GC, Bestagno M, Cerino A, *et al*: Antibodies to hnRNP core protein A1 in connective tissue diseases. J Cell Biochem 40:43–47, 1989

Bach JF, Koutouzov S, Endert PMv: Are there unique autoantigens triggering autoimmune diseases. Immunol Rev 164:139–155, 1998

Chou YK, Bourdette DN, Offner H, Whitham R, Wang RY, Hashim GA, Vandenbark AA: Frequency of T cells specific for myelin basic protein and myelin proteolipid protein in blood and cerebrospinal fluid in multiple sclerosis. J Neuroimmunol 38:105–113, 1992

Gudmundsdottir AS, Sigmundsdottir H, Sigurgeirsson B, Good MF, Valdimarsson H, Jonsdottir I: Is an epitope on keratin 17 a major target for autoreactive T lymphocytes in psoriasis? Clin Exp Immunol 117:580–586, 1999

Harrison LC, Honeyman MC, DeAizpurua HJ, Schmidli RS, Colman PG, Tait BD, Cram DS: Inverse relation between humoral and cellular immunity to glutamic acid decarboxylase in subjects at risk of insulin-dependent diabetes. Lancet 341:1365–1369, 1993

Hellings N, Baree M, Verhoeven C, *et al*: T-cell reactivity to multiple myelin antigens in multiple sclerosis patients and healthy controls. J Neurosci Res 63:290–302, 2001

Hueber W, Utz PJ, Steinman L, Robinson WH: Autoantibody profiling for the study and treatment of autoimmune disease. Arthritis Res 4:290–295, 2002

Hummel M, Durinovic-Bello I, Ziegler AG: Relation between cellular and humoral immunity to islet cell antigens in type 1 diabetes. J Autoimmun 9:427–430, 1996

Kallioinen M, Koivukangas V, Jarvinen M, Oikarinen A: Expression of cytokeratins in regenerating human epidermis. Br J Dermatol 133:830–835, 1995

Kawasaki E, Eisenbarth GS: High-throughput radioassays for autoantibodies to recombinant autoantigens. Front Biosci 5:E181–E190, 2000

KerlerodeRosbo N, Hoffman M, Mendel I, *et al*: Predominance of the autoimmune response to myelin oligodendrocyte glycoprotein (MOG) in multiple sclerosis: Reactivity to the extracellular domain of MOG is directed against three main regions. Eur J Immunol 27:3059–3069, 1997

Krebs P, Kurrer M, Sahin U, Tureci O, Ludewig B: Autoimmunity seen through the SEREX-scope. Autoimmun Rev 2:339–345, 2003

Lacroix-Desmazes S, Kaveri SV, Mouthon L, Ayouba A, Malanchere E, Coutinho A, Kazatchkine MD: Self-reactive antibodies (natural autoantibodies) in healthy individuals. J Immunol Methods 216:117–137, 1998

Lernmark A: Autoimmune diseases: Are markers ready for prediction. J Clin Invest 108:1091–1096, 2001

Leslie RD, Atkinson MA, Notkins AL: Autoantigens IA-2 and GAD in Type I (insulin-dependent) diabetes. Diabetologia 42:3–14, 1999

Leslie D, Lipsky P, Notkins AL: Autoantibodies as predictors of disease. J Clin Invest 108:1417–1422, 2001

Levin MC, Lee SM, Kalume F, *et al*: Autoimmunity due to molecular mimicry as a cause of neurological disease. Nat Med 8:509–513, 2002

Lovett-Racke AE, Trotter JL, Lauber J: Decreased dependence of myelin basic protein-reactive T cells on CD28-mediated costimulation in multiple sclerosis patients. A marker of activated/memory T cells. J Clin Invest 101:725–730, 1998

Lukyanov K, Diatchenko L, Chenchik A: Construction of cDNA libraries from small amounts of total RNA using the suppression PCR effect. Biochem Biophys Res Commun 230:285–288, 1997

Maclaren N, Lan M, Coutant R, *et al*: Only multiple autoantibodies to islet cells (ICA), insulin, GAD65, IA-2 and IA-2beta predict immune-mediated (Type 1) diabetes in relatives. J Autoimmun 12:279–287, 1999

McCutcheon M, Wehner N, Wensky A: A sensitive ELISPOT assay to detect low-frequency human T lymphocytes. J Immunol Methods 210:149–166, 1997

Mocci S, Lafferty K, Howard M: The role of autoantigens in autoimmune disease. Curr Opin Immunol 12:725–730, 2000

Montecucco C, Caporali R, Negri C, *et al*: Antibodies from patients with rheumatoid arthritis and systemic lupus erythematosus recognize different epitopes of a single heterogeneous nuclear RNP core protein. Possible role of cross-reacting antikeratin antibodies. Arthritis Rheum 33:180–186, 1990

Narendran P, Williams AJ, Elsegood K, Leech NJ, Dayan CM: Humoral and cellular immune responses to proinsulin in adults with newly diagnosed type 1 diabetes. Diabetes Metab Res Rev 19:52–59, 2003

Old LJ, Chen YT: New paths in human cancer serology. J Exp Med 187:1163–1167, 1998

Pedersen IB, Knudsen N, Jorgensen T, Perrild H, Ovesen L, Laurberg P: Thyroid peroxidase and thyroglobulin autoantibodies in a large survey of populations with mild and moderate iodine deficiency. Clin Endocrinol (Oxf) 58:36–42, 2003

Pelfrey CM, Rudick RA, Cotleur AC, Lee JC, Tary-Lehmann M, Lehmann PV: Quantification of self-recognition in multiple sclerosis by single-cell analysis of cytokine production. J Immunol 165:1641–1651, 2000

Plotz PH: The autoantibody repertoire: Searching for order. Nat Rev Immunol 3:73–78, 2003

Robinson C, Callow M, Stevenson S, Scott B, Robinson BW, Lake RA: Serologic responses in patients with malignant mesothelioma: Evidence for both public and private specificities. Am J Respir Cell Mol Biol 22:550–556, 2000

Robinson WH, DiGennaro C, Hueber W, *et al*: Autoantigen microarrays for multiplex characterization of autoantibody responses. Nat Med 8:295–301, 2002

Ruf J, Ferrand M, Durand-Gorde JM, Carayon P: Immunopurification and characterization of thyroid autoantibodies with dual specificity for thyroglobulin and thyroperoxidase. Autoimmunity 11:179–188, 1992

Sahin U, Tureci O, Schmitt H, *et al*: Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci USA 92:11810–11813, 1995

Stoler A, Kopan R, Duvic M, Fuchs E: Use of monospecific antisera and cRNA probes to localize the major changes in keratin expression during normal and abnormal epidermal differentiation. J Cell Biol 107:427–446, 1988

Tejada-Simon MV, Hong J, Rivera VM, Zhang JZ: Reactivity pattern and cytokine profile of T cells primed by myelin peptides in multiple sclerosis and healthy individuals. Eur J Immunol 31:907–917, 2001

Tureci O, Sahin U, Pfreundschuh M: Serological analysis of human tumor antigens: Molecular definition and implications. Mol Med Today 3:342–349, 1997

Tureci O, Sahin U, Zwick C, Neumann F, Pfreundschuh M: Exploitation of the antibody repertoire of cancer patients for the identification of human tumor antigens. Hybridoma 18:23–28, 1999

van Muijen GN, Ruiter DJ, Franke WW, Achtstatter T, Haasnoot WH, Ponec M, Warnaar SO: Cell type heterogeneity of cytokeratin expression in complex epithelia and carcinomas as demonstrated by monoclonal antibodies specific for cytokeratins nos 4 and 13. Exp Cell Res 162: 97–113, 1986

van Muijen GN, Warnaar SO, Ponec M: Differentiation-related changes of cytokeratin expression in cultured keratinocytes and in fetal, newborn, and adult epidermis. Exp Cell Res 171:331–345, 1987

van Rossum MM, Mommers JM, van de Kerkhof PC, van Erp PE: Coexpression of keratins 13 and 16 in human keratinocytes indicates association between hyperproliferation-associated and retinoid-induced differentiation. Arch Dermatol Res 292:16–20, 2000a

van Rossum MM, Mommers JM, van Erp PE, Leyninger E, Clucas A, van de Kerkhof PC: CD 2394, a novel synthetic retinoid, initiates an embryonic type of differentiation in hyperproliferative skin. Acta Dermatol Venereol 80:98–101, 2000b

Viglietta V, Kent SC, Orban T, Hafler DA: GAD65-reactive T cells are activated in patients with autoimmune type 1a diabetes. J Clin Invest 109:895–903, 2002

von Herrath MG, Harrison LC: Antigen-induced regulatory T cells in autoimmunity. Nat Rev Immunol 3:223–232, 2003

Zhang J, Markovic-Plese S, Lacet B, Raus J, Weiner HL, Hafler DA: Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. J Exp Med 179:973–984, 1994